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SCE AND MEIOTIC CROSSOVER EXCHANGE IN GERM CELLS

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ABSTRACT

In vivo BrdU-dye methodology has enabled greatly improved analyses of sister chromatid exchange (SCE) and meiotic (non-sister) crossover exchange in germ cells. Mouse spermatogonial cells can be evaluated for SCE induction after *in vivo* exposure to chemical mutagens and carcinogens. In this system cyclophosphamide and ethyl carbamate have been shown to cause significant increases in SCE which, however, tend to be lower in magnitude than those expressed by various somatic tissues. Armenian hamster meiotic chromosomes can be evaluated for normal levels of SCE, and both quantitative and qualitative aspects of crossover exchange. Alternate crossover exchange patterns appear directly related to chiasmata in primary spermatocyte sex bivalents, and also are detectable in sex chromosomes from secondary spermatocytes. Thus, high resolution chromatid exchange analysis is versatile in its application for studies of various forms of normal and abnormal genetic recombination processes in mammalian germ cells.

INTRODUCTION

Meiotic crossing over and sister chromatid exchange (SCE) each represent a distinctive form of DNA recombination. Yet their concurrence in germ cells and detection by similar methodologies lend them to comparative analyses. Although both processes involve reciprocal chromatid exchange, their mechanics and significance are fundamentally different. Crossovers are exchanges between non-sister chromatids of homologous chromosomes. In general, they are considered to occur after the main period of DNA synthesis (in prophase), and involve delayed and also repair synthesis (1). They appear localized to precise sites along synapsed maternal- and paternal-derived meiotic chromosomes. Chiasmata have traditionally been inferred to arise from, and directly manifest, crossover exchange. While a complete molecular explanation for crossing over is lacking (for diseussion, see Holliday, 1977 [2]), its contribution to the endowment of genetic variability upon germ cells is nonetheless central to classical concepts of heredity.

In contrast, SCEs are irregularly occurring intrachromosomal events that arise during DNA synthesis in mitotic (3) and meiotic chromosomes. Unlike crossing over, there does not appear to be any resulting alteration in genetic content at the chromosome level. The meaning and frequency of SCE as a normal process is not clear. Equally uncertain is its mechanism of formation and extent of molecular relationship to crossing over. Yet, SCE has taken on special importance as a sensitive index of damage from exposure to genotoxic agents (for recent reviews, see Perry, 1980 [4]; Latt et al., 1980 [5]).

Historically, symmetrical sister and non-sister chromatid exchanges were directly detected with autoradiography. In landmark studies with plants and grasshoppers, J.H. Taylor and associates determined that radiolabeled DNA is semi-conservatively distributed in mitosis (6) and meiosis (7). The incorporation of [^{3}H]dT during DNA synthesis results in differentially-labeled sister chromatids at the second subsequent metaphase stage. Exchanges are then signaled by switches in chromatid labeling patterns. H. Kato (8) and S. Wolff et al. (3) used this methodology to detect an increase in the frequency of SCE in mammalian somatic cells exposed in vitro to mutagens and carcinogens. Due to poor growth of germ cells in culture, as well as other technical constraints, there has been little attention to possible mutagen effects upon either form of chromatid exchange in germ tissue. A few in vivo studies have noted heat influences to reduce chiasma frequencies in grasshoppers (9) and chemical effects to produce univalents or to interfere with chiasma formation in animals (10,11) and plants (12). However, the general inability to resolve label exchanges between closely adhered (synapsed) meiotic chromatids has precluded direct autoradiographic assessments of either normal or abnormal crossover exchange products. Analyses of secondary spermatocytes, typically insect (7,9,13), have been performed. These metaphase stages are one cell division removed from crossing over and chiasma formation, and the interpretation of label patterns is complicated by the occurrence of both SCE and crossover products. Consequently, only limited determinations of normal frequencies and cytological features of sister and non-sister chromatid exchange in meiotic cells have been possible.

The development of 5-bromodeoxyuridine (BrdU)-fluorescent-dye methodology by S. Latt (14,15) has provided a new potential for expedient analyses of SCE, and unequivocal identification of crossover exchange. With this technique, and the important option of permanent Giemsa staining (16), sister chromatids are differentiated through eventual asymmetry in substitution of BrdU for thymidine, and staining with a BrdU-sensitive dye. Exchange points are delineated as abrupt discontinuities in chromatid stain patterns. In vivo studies in mammals with this technique were slow to develop because the liver rapidly degrades BrdU (17) and deters its incorporation into DNA. However, early methods which continually replenished BrdU to rodents achieved differential staining of both mitotic (spermatogonial) (18) and meiotic (spermatocyte) (19) chromatids. Spermatogonial cells examined from mice injected with genotoxic agents revealed germ tissue-specific levels of increase in SCE frequency (18,20,21). Systems accomplishing the characterization of SCE and crossover exchange in meiotic chromosomes have now been reported in locusts (22,23), hamsters (20,24), and mice (25-27). These schemes allow unprecedented studies into the fundamental nature of meiotic crossovers, especially concerning breakage and reunion phenomena and chiasmata. In the following text, a basic differential staining approach for analyses of SCE and crossover exchange in germ cells of male rodents is described, and results to date are summarized.

METHODOLOGY

The requirement for sister chromatid differentiation is common to SCE and crossover analyses, and in principle, is accomplished in an identical manner. In vivo BrdU methodology to achieve differential staining of mitotic and

meiotic sister chromatids depends upon sequential steps of DNA labeling, cell cycling, and chromosome staining (Figure 1). Adult male rodents (i.e., mice, hamsters) are treated with a prolonged exposure to BrdU in order to effect analog substitution during germ cell DNA synthesis. Germ cells appear to be more susceptible than somatic cells to toxic effects from BrdU. However, techniques relying upon analog administration by serial i.p. injections (18), infusion (29), s.c. tablet implantation (20), and i.p. injection of a charcoal-analog suspension (30) have proven effective. BrdU substitution over only the first of two consecutive replications is sufficient to obtain asymmetrically labeled, and differentially stained, sister chromatids at the second metaphase stage. Labeling over the second DNA synthesis period is optional: levels of analog substitution differ accordingly, although the quality of chromatid contrast resulting after application of a BrdU-sensitive dye (fluorescent [14] or fluorescent plus Giemsa [16]) is similar. In SCE induction studies, the test chemical has usually been injected intraperitoneally at a time judged to coincide with the beginning of the second DNA synthesis period.

Figure 2 illustrates germ cell progression through mitosis and meiosis, and the context in which BrdU methodology is applied for studies of spermatogonial SCE and spermatocyte SCE and crossover exchange. The analysis of exchange in either cell type requires BrdU substitution into spermatogonial DNA, although the specific replication periods involved are different. Since germ cell renewal is continuous and asynchronous (31), an <u>in vivo</u> BrdU treatment effects simultaneous labeling over DNA synthesis periods at various stages of spermatogenic development. Testicular cells harvested approximately

$2^1/2$ days after BrdU treatment reveal second division sister chromatid differentiation and exchange restricted to spermatogonial cells. However, when cell harvest is delayed for approximately 2 weeks after BrdU treatment, chromosomes labeled over the penultimate DNA synthesis period will have progressed through a lengthy meiotic prophase I period and arrived at metaphase stages. As there is no intervening DNA synthesis period between primary and secondary spermatocytes (the latter formed by reductive division), similar second division differential stain patterns characterize meiotic metaphase I and metaphase II chromatids. Exchanges evident may reflect SCE occurring over either of the two preceding DNA synthesis periods, or crossing over during primary spermatocyte prophase. As discussed below, crossover exchanges are directly distinguishable by isolabeling for involved chromosome regions.

SISTER CHROMATID EXCHANGE IN SPERMATOGONIA

Mouse spermatogonial cells reveal low levels of SCE (Figure 3). Average values of about 1.8 SCEs per diploid cell have been recorded for more than 500 genomes after normalization for the many multiple genome figures characteristic of male germinal tissue (18,19). Although spermatogonial subpopulations were not distinguished, it is presumed that the "type b" cell which is most prevalent in the testis (31,32) also is most abundantly represented in the chromosome analyses. The baseline SCE frequencies are consistent among various modes of <u>in vivo</u> BrdU labeling employed, except that higher values (approx. 3 SCEs per cell) are generally experienced with BrdU tablet methodology (20,21). Regardless of specific technical methods used, spermatogonial SCE frequencies are usually lower than those of various somatic

tissues measured for comparison. There is some disagreement between studies concerning relative marrow and spleen values (20,21,29,33); however, thymus and liver (20,21), and intestine, lymph node, and ascites tumor cells (29,30) reportedly have higher baseline values -- in some instances, more than four times greater than that of spermatogonia.

An important potential of spermatogonial SCE analysis in mutagen- or carcinogen-exposed animals is to confirm the distribution of genetically active substances to the germ tissue, and to determine the relative level of DNA reactivity. After intraperitoneal exposure of mice to mitomycin C (18), cyclophosphamide (19,20), and ethyl carbamate (21), dose-dependent increases in spermatogonial SCE levels have been demonstrated. Cyclophosphamide and ethyl carbamate are negative or weak inducers of SCE in rodent cell cultures (19,21,34,35). After in vivo exposure and host activation, these chemicals cause up to fivefold increases in the baseline level of spermatogonial SCE. Yet, these increases tend to be proportionally lower than those experienced by somatic cells from similarly treated mice (Table 1). For example, 400 mg/kg ethyl carbamate causes less than a doubling of SCE in spermatogonia while bone marrow and regenerating liver cells reveal nearly sixfold increases over the baseline level of SCE.

As noted elsewhere (20,21,36), possible explanations for the disparate somatic versus germ-tissue SCE values include differences in cell cycling, metabolic capabilities, DNA repair, BrdU effects, and pharmacokinetic factors resulting in an unequal distribution of active metabolites. Painter (37) has proposed that SCEs may arise from unrepaired lesions localized at junctions between replicon clusters. The duration of DNA synthesis in spermatogonial

cells is typically about twice that of the aforementioned somatic tissues (32,38). This marked discordance in rates of DNA synthesis suggests the possibility of major organizational differences at the replicon level. Perhaps features pertaining to replicon size and/or activity unique to germ cells confer a reduction in the number, or sensitivity, of sites vulnerable to SCE formation.

SISTER CHROMATID EXCHANGE IN MEIOTIC CELLS

Prior to the realization of its practical potential for signaling genotoxic effects in mitotic chromosomes, SCE in germ cells was almost always studied in meiotic chromosomes. Early evidence for spontaneous SCE formation came from Drosophila (39) and maize (40) studies in which SCE was considered implicated in ring and rod chromosome configurations. One autoradiographic analysis in grasshoppers (41) revealed a binucleate spermatocyte possessing a specific chromosomal SCE in only one of the nuclei, thereby denoting its ability to form during unique (1) pre-meiotic DNA synthesis. Additional autoradiography studies in grasshopper secondary spermatocytes noted the confusion surrounding interpretations of SCE and crossover exchange due to instances of possible mimicry in appearance. SCE frequencies were calculated after indirect accounting of crossovers projected from chiasma frequencies. Some conflicting results were obtained regarding the interpretation of SCE as a rare (7,42) or common (9) event.

A recent BrdU-dye analysis of locust primary spermatocytes has revealed baseline SCE frequencies ranging from approximately 0.3 to 0.9 for specific bivalents (43). General randomness in distribution, and also frequency

dependence upon chromosome length, was noted. Similar studies in mammals have disclosed lower values, although it is not known whether these differences relate to the organisms or to the BrdU exposure dose inside (15) or outside (44) the DNA. Respective values of approximately 0.10 and 0.07 SCEs/bivalent obtained for female (27) and male (26) mice are generally consistent with the spermatogonial frequencies of < 2 SCEs/diploid cell noted earlier. Similarly, in 233 sex bivalents thus far analyzed from male Armenian hamsters (Figure 4), SCE has been detected with a frequency of 0.15/bivalent. To what extent, if any, there may be any fundamental differences in SCE frequency or distribution occurring in the pre-meiotic, as opposed to the last mitotic, DNA synthesis period remains questionable.

CROSSOVER EXCHANGE

Crossing over has traditionally been recognized by the appearance of a chiasma. This chromosomal configuration was interpreted as arising from the recombination of homologous non-sister chromatids followed by persistent pairing of former (pre-crossover) sister chromatid regions. That homologs are broken and reunited was shown with autoradiographic studies, primarily of insect meiosis, wherein exchanges between differentially labeled non-sister chromatids were detected (7,9,42). Higher resolution BrdU-dye analyses in insects (23) and rodents (24-26) have recently confirmed these findings and unequivocally localized the sites of exchange to the chiasma. Together, these studies in primary spermatocytes and oocytes have supported major tenets of classical chiasmatype theory (45).

In autoradiographic studies with the grasshopper (7), Taylor noted that half of meiotic crossovers should be "hidden" due to random involvement of like-labeled non-sister chromatids in the exchange process. Only exchanges involving unlike-labeled non-sister chromatids result in "visible" crossovers. BrdU-dye analyses of recombination products in locusts (23), hamsters (24), and mice (25,26) are in complete accord with this principle. Large numbers of differentially stained bivalents in these species have conformed to an approximate 1:1 ratio of hidden versus visible chromatid exchanges across the chiasma. In the Armenian hamster system, primary spermatocytes with sister chromatid differentiation in all chromosomes are analyzable (24). However, the unusual monochiasmate sex bivalent (46,47) is particularly well suited for qualitative and quantitative assessments of crossing over. The sex chromosomes in this species are further distinguished by their individual morphologies (46) and their considerable asynchrony in replication which often highlights them exclusively with clear differential staining (24).

In Figure 5, the alternative types of crossover exchange between X and Y chromatids are diagrammed and accompanied by cytological examples at diakinesis-metaphase I stages. In "a," hidden crossing over is exemplified by continuous chromatid staining into the chiasma. There is no cytological evidence of chromatid recombination other than that indirectly inferred by the presence of the chiasma. In "b," isolabeling for bright or dull short-arm regions on either side of the chiasma provides visible evidence of meiotic crossing over between the X and Y chromosomes. In all three bivalents, it is apparent that a bright chromatid short-arm segment from one sex chromosome underwent a reciprocal exchange of position with a dull homologous segment on

the other sex chromosome. In 141 sex bivalents from two young male Armenian hamsters, the ratio of hidden to visible forms of crossover was 53:47%, respectively (24).

The sex bivalent examples in Figure 5b address additional features of the recombination process as well. Sister chromatid pairing across the chiasma dictates that recombined chromatids should orient in diagonal apposition, and likewise for unrecombined chromatids. Yet, in the center bivalent of Figure 5b, parallel orientations across the chiasma are evident. This anomalous type of exchange is similar to that described for the locust (23) and mouse (26). It is infrequently observed, and may theoretically be explained by the occurrence of SCE in the crossover region or by combinations of crossovers and SCEs (23,24,26). The sex bivalent to the left (Figure 5b) clearly reveals that the point of exchange coincides with the proximal boundary of paired chromatid segments forming the chiasma. This type of precise correspondence between visibly stained crossovers and chiasmata has been observed in the insect (23) and rodent (24-26), and clearly supports their synonymy in representation of the same pheneomenon. The bivalent to the right gives the impression of chiasma terminalization movement (45) by its end-to-end association of X and Y chromatids distal to the visible crossover site. However, in a large number of monochiasmate bivalents from the locust, no evidence for chiasma terminalization has been obtained (23). It has been pointed out that the chromatids for the region in question within the illustrated hamster bivalent are splayed apart (as if an artifact of preparation) rather than intimately paired as might be expected for true chiasma terminalization (48). Other bivalents (not shown) have in fact

conformed closer to this supposedly more correct image. In the mouse, no clear examples of chiasma terminal movement have been obtained (26,27), although a few isolated instances of what appeared to be terminalization were noted (27). The balance of observations in these systems favors a stand against the basic concept of chiasma terminalization. However, the suspiciously common Armenian hamster bivalents with homolog associations resembling terminalized chiasmata warrant further study. As pointed out by Polani and colleagues (27), this issue is interwoven with broader considerations into the nature of sister chromatid cohesiveness at meiosis and the possible implication of axial elements in this function.

Chromatid regions undergoing crossover exchange are thus clearly defined by stretches of isolabeling in association with chiasmata. The new technical capability to view this has opened the way for studies into various aspects of crossing over. Jones and Tease have looked for chromatid interference in locust dichiasmate bivalents, and found no evidence from observed hidden and visible exchange frequencies to indicate that involvement of a chromatid in one crossover influences the likelihood of its involvement in another crossover (49). Other crossover studies include those concerned with age effects (24), the relative timing of semi-conservative DNA replication in the region of exchange (24), and the chiasmate versus achiasmate status or history of univalents and bivalents revealing only terminal chromatid associations (26). The diversity of studies in the primary spermatocyte or oocyte stages underscores the importance of methodological advances that permit the distinction of crossover exchange from SCE. As noted below, the value of chromatid recombination analyses in meiotic metaphase II stages is

limited by the possibility of misinterpreting stain patterns generated by combinations of crossovers and SCEs.

X and Y chromosomes segregate at anaphase I and display their unchanged stain patterns at metaphase II of haploid secondary spermatocytes. Representative Y chromosomes with various stain images are illustrated in Figure 6. The cell in Figure 6a reveals the generally late-replicating Y chromosome highlighted with sister chromatid differentiation in the long arms. Isolabeling for bright short-arm regions is consistent with that expected from an earlier visible-type mode of crossing over. Similarly, isolabeling for dull short-arm regions (Figure 6b) is detectable in other Y-bearing cells. Y chromosomes with a history of hidden-type crossing over should reveal sister chromatid differentiation (Figure 6c) in which SCE images may also be detected (Figure 6d). However, without the ability to orient stain patterns to chiasmata, hidden-type crossovers are not distinguishable from an absence of crossing over. Moreover, as comprehensively discussed by Jones and co-workers (13,42), patterns accurately reflecting specific types of exchange may be confused with those generated by various combinations of SCE and crossing over. This is more of a problem in dichiasmate bivalents where, for example, a proximal hidden-type crossover will change a distal visible crossover stain pattern to one resembling SCE (13). Despite these limitations, metaphase II cell analyses offer some advantages in their clear chromosome morphology and staining. In the Armenian hamster system, these cells have proven useful in combined analyses with primary spermatocytes for evaluating chromatid exchange frequencies in sex chromosomes and for more precisely localizing sites of crossover exchange in autosomes (24).

SUMMARY

Differential staining methodology is advantageously applied for versatile studies of genetic recombination in germ cells. BrdU-labeled spermatogonial cells may be harvested at mitotic metaphase stages for the evaluation of SCE, or they may be allowed to mature into spermatocytes wherein both SCEs and crossover exchanges are analyzable. SCE induction by host-activated chemical mutagens and carcinogens has been detected in spermatogonial cells. Sister and non-sister chromatid exchanges have become clearly distinguishable from one another and directly quantifiable in mammalian meiotic chromosomes. In the very few insect and rodent analyses performed to date, significant advances have been made towards the experimental confirmation of, or challenge to, classical genetic theories. The direct relationship of crossover exchange to chiasma formation is strongly supported, while concepts of chiasma terminalization and chromatid interference have come under serious question.

There appears to be an important new potential attached to cytogenetic approaches for the evaluation of molecular and cytological features of SCE and crossover exchange formation. The determination of possible mutagen effects upon crossover exchange is likely to be far more complex than that of SCE induction. However, future studies directed to this question may prove equally rewarding.

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LEGENDS

Figure 1. Diagrammatic representation of in vivo protocol for effecting sister chromatid differentiation in mitotic and meiotic chromosomes. Unsubstituted DNA chains are represented by solid lines and BrdU-substituted chains by broken lines. 33258 Hoechst fluorescence is denoted as bright by black shading and dull by grey shading. Following BrdU incorporation for one DNA synthesis period, first-division chromosomes are unifilarly substituted in each chromatid and exhibit dull fluorescence (due to a BrdU quenching effect) in chromatid arms. Subsequent replication without BrdU incorporation results in second-division chromosomes with asymmetrical BrdU substitution. Bright fluorescence of unsubstituted chromatids contrasts with dull fluorescence of chromatids with single-strand substitution (28).

Figure 2. Chromatid exchange analyses in male germ cells. BrdU labeling over pre-meiotic DNA replication periods permits analyses of SCE in spermatogonial mitotic chromosomes, and of SCE and crossover exchange in spermatocyte meiotic chromosomes. As indicated, different requirements for these analyses pertain to the particular replication period undergoing BrdU substitution, and the time of cell harvest. Further explanation is provided in the text.

Figure 3. Sister chromatid differentiation and exchange (arrows) in a mouse spermatogonial cell after in vivo administration of BrdU. In "a," the cell is stai.ed with 33258 Hoechst fluorescent dye. Its appearance in "b" is after restaining with Giemsa dye (18).

Figure 4. Sister chromatid differentiation and exchange in the sex bivalent of an Armenian hamster spermatocyte. Two SCEs are evident (arrows) in the long arm of the Y chromosome. (Isolabeling in the chiasmatic short arms of X and Y chromosomes is indicative of crossover exchange, while that in the distal long arms of the X stems from asynchronous replication.) (24)

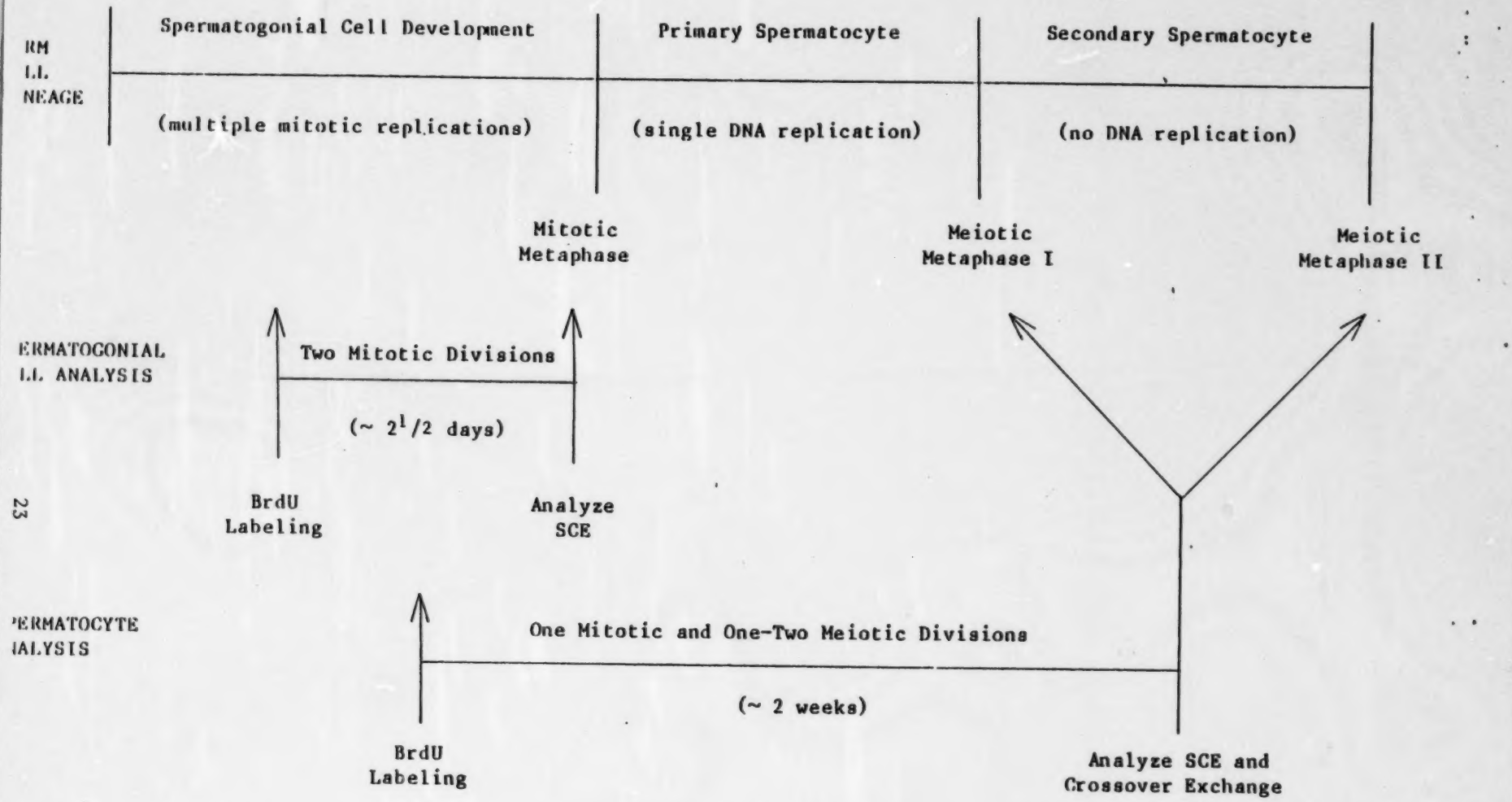
Figure 5. Alternative types of crossing over between meiotic sex chromosomes. In "a," crossover formation between like-stained non-sister chromatids is diagrammed. Examples of Armenian hamster sex bivalents at subsequent diakinesis-metaphase I stages, assuming pairing is between chromatin previously found on sister chromatids, present no visible evidence of exchanged short-arm segments near the chiasma. In "b," crossing over involves unlike-stained non-sister chromatids: recombinant regions appear isolabeled in the cytological examples (24).

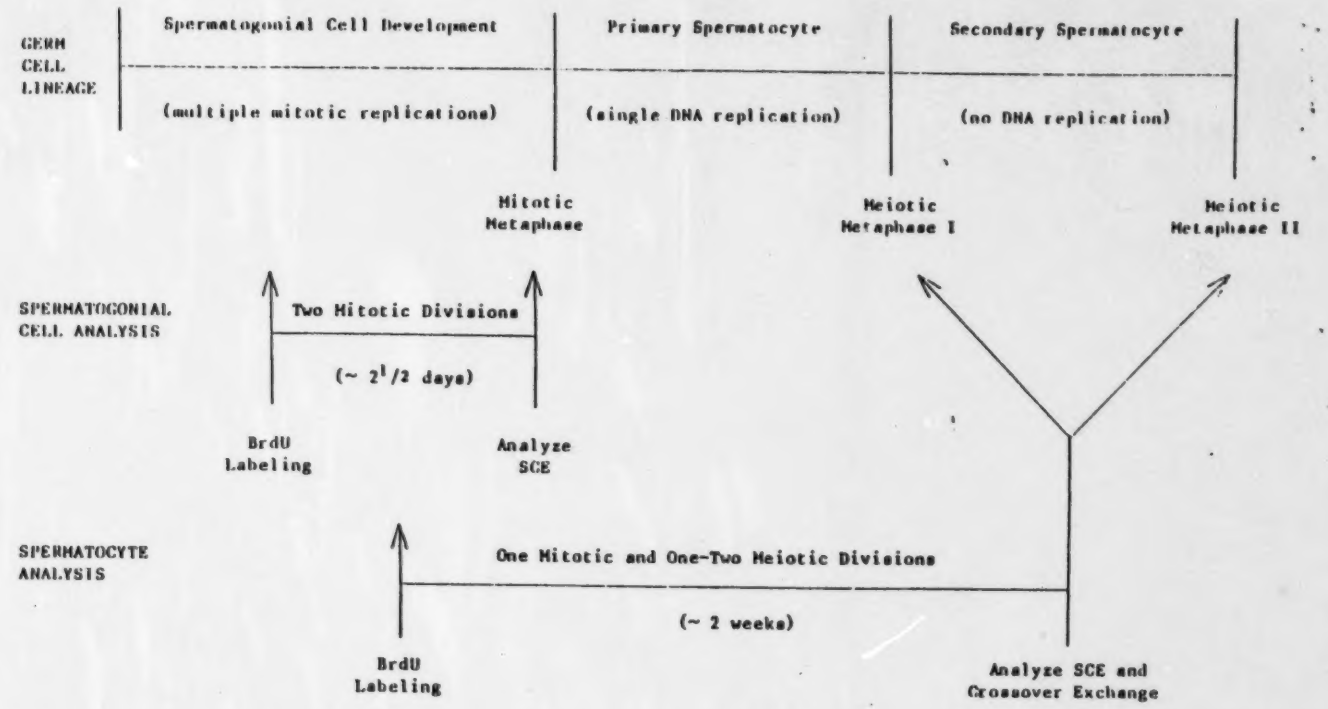
Figure 6. Alternative stain patterns in Y chromosomes from different secondary spermatocytes. The haploid metaphase II stage in "a" reveals isolabeled bright short-arm regions in the Y (arrow), as if it segregated from a bivalent characterized by visible crossing over. Y short-arm patterns of dull isolabeling in "b," sister chromatid differentiation in "c," and apparent SCE in "d" portray those expected following a visible crossover, a hidden crossover, and a hidden crossover + SCE, respectively.

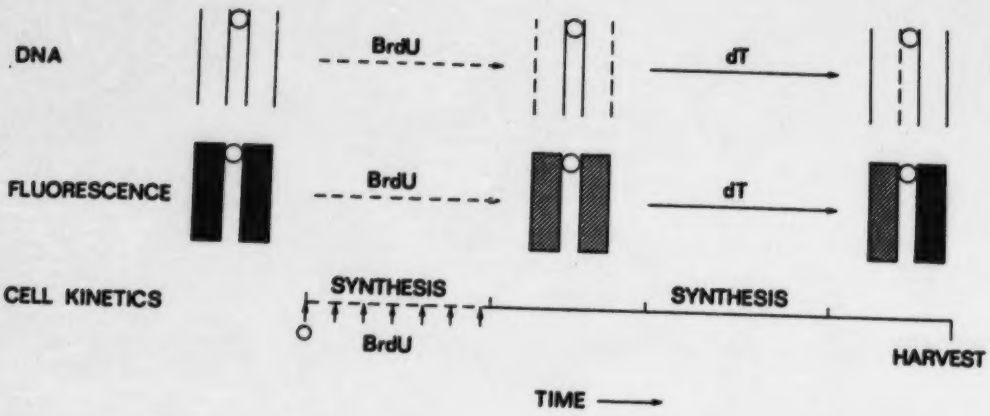
Table 1. Tissue-Specific SCE Frequencies in Mice Exposed to Cyclophosphamide or Ethyl Carbamate[a]

Tissue	Controls SCEs/cell[b]	Cyclophosphamide-5 mg/kg SCEs/cell	(Increase)[c]	Ethyl Carbamate-400 mg/kg SCEs/cell	(Increase)
Spermatogonia	3.4 ± 0.2	8.1 ± 0.6	(2.4X)	-	-
Bone marrow	7.4 ± 0.2	22.4 ± 0.6	(3.0X)	-	-
Spleen	6.8 ± 0.4	22.0 ± 1.6	(3.2X)	-	-
Thymus[d]	9.1 ± 0.4	33.1 ± 2.2	(3.6X)	-	-
Spermatogonia	2.2 ± 0.1	-	-	3.8 ± 1.3	(1.7X)
Bone marrow	7.7 ± 3.0	-	-	44.1 ± 10.6	(5.7X)
Liver[e]	9.9 ± 3.4	-	-	56.1 ± 13.3	(5.7X)

[a]Data are compiled from (20) and (21).
[b]Mean ± standard error per sample of from 24 to 262 cells.
[c]Proportional increase in SCE over respective control level.
[d]Thymus from male one-month-old CBA/J mice; other tissues from male three-month-old CBA/J mice. All mice were implanted with a BrdU tablet.
[e]Liver is regenerating after partial hepatectomy.


RM
LL
NEAGE
Spermatogonial Cell Development
Primary Spermatocyte
Secondary Spermatocyte
(multiple mitotic replications)
(single DNA replication)
(no DNA replication)
Mitotic
Metaphase
Meiotic
Metaphase I
Meiotic
Metaphase II
ERMATOGONIAL
LL ANALYSIS
Two Mitotic Divisions
(~ 2 1/2 days)
BrdU
Labeling
Analyze
SCE
ERMATOCYTE
NALYSIS
One Mitotic and One-Two Meiotic Divisions
(~ 2 weeks)
BrdU
Labeling
Analyze SCE and
Crossover Exchange


GERM CELL LINEAGE
Spermatogonial Cell Development
Primary Spermatocyte
Secondary Spermatocyte
(multiple mitotic replications)
(single DNA replication)
(no DNA replication)
Mitotic Metaphase
Meiotic Metaphase I
Meiotic Metaphase II
SPERMATOGONIAL CELL ANALYSIS
Two Mitotic Divisions
(~ 2 1/2 days)
BrdU Labeling
Analyze SCE
SPERMATOCYTE ANALYSIS
One Mitotic and One-Two Meiotic Divisions
(~ 2 weeks)
BrdU Labeling
Analyze SCE and Crossover Exchange



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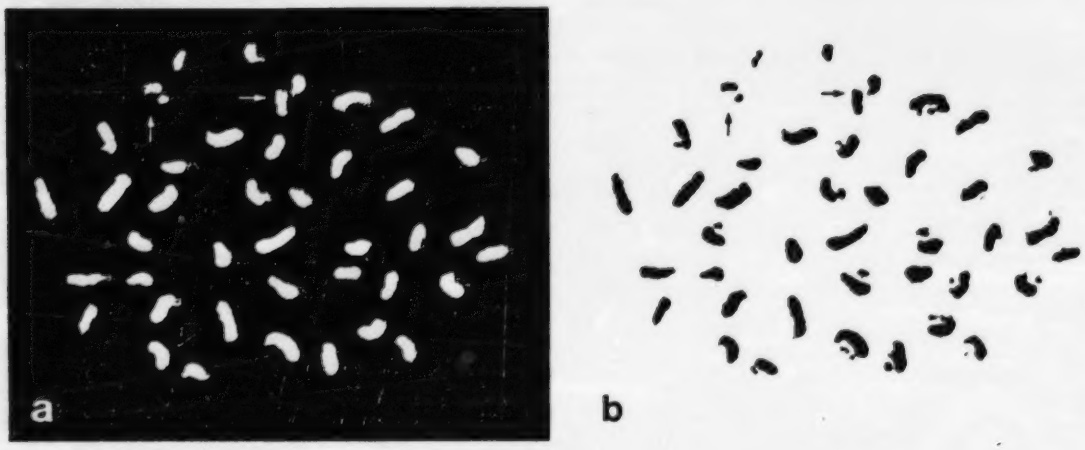

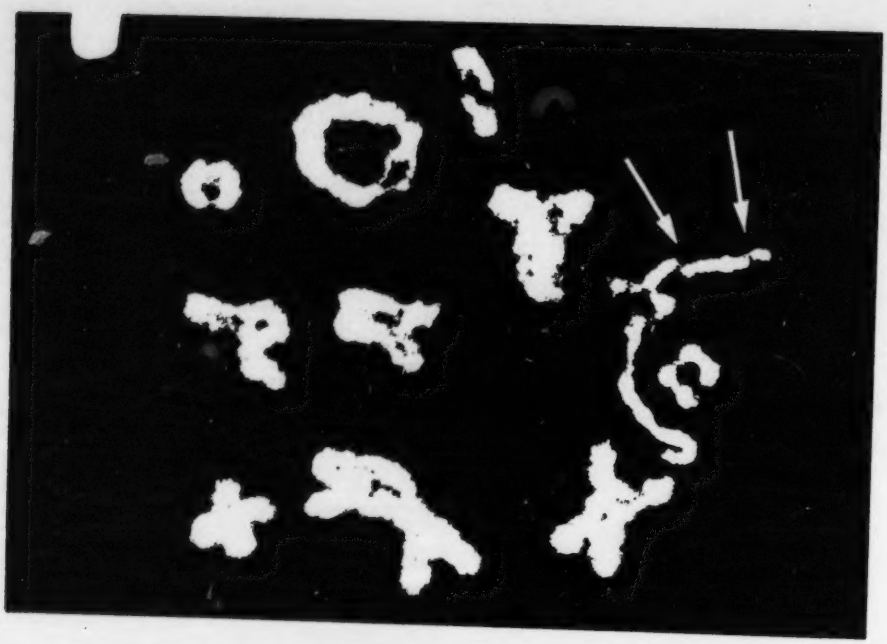

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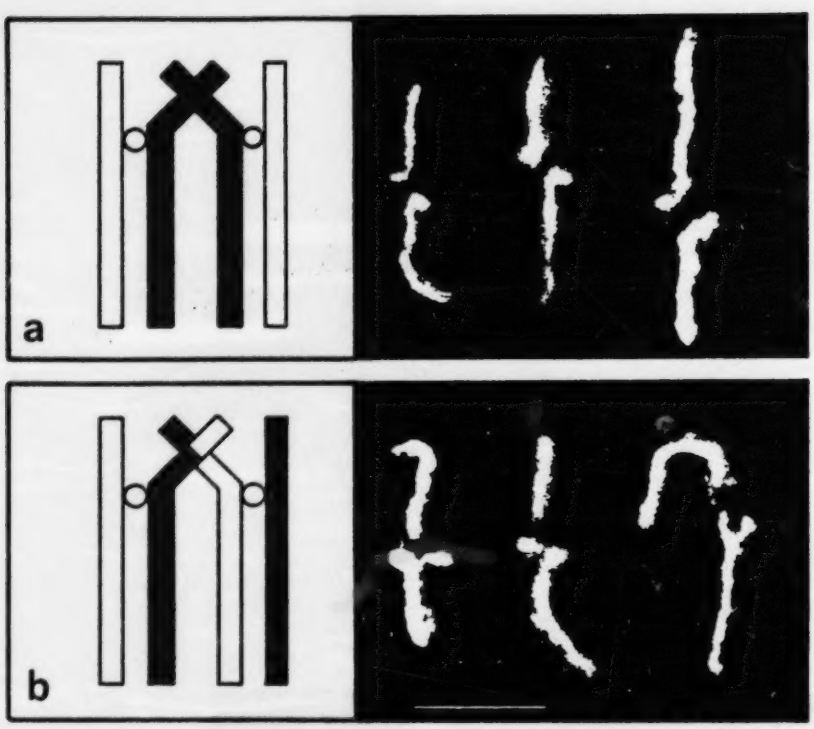

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